

The lateral hypothalamic parvalbumin-immunoreactive (PV1) nucleus in rodents*

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*Dedicated to Emilio Celio (1927-2011), founder of Swant.

Abbreviations:

Anatomic:

IIn: optic nerve

3dV: third ventricle

A: amygdala
AHA: anterior hypothalamic area
Cer: cerebellum
cp: cerebral peduncle
DMH: dorsomedial hypothalamic nucleus
DHA2: part of the dorsal hypothalamic area
EP: entopeduncular nucleus
fx: fornix
GP: globus pallidus
IHA1: part of the intermediate hypothalamic area
I: infundibulum
IP: interpeduncular fossa
ISM: interstitial nucleus of the stria medullaris
LHN1 and LHN2: part of the lateral hypothalamic nucleus
LHN4: part of the lateral hypothalamic area
LHAL: anterolateral subarea of the lateral hypothalamic area (Swanson, 2004)
LHAav: lateral hypothalamic area, anterior region, ventral zone (Swanson, 2004)
LHApc: lateral hypothalamic area, parvicellular region (Swanson, 2004)
LHVL1: part of the ventrolateral subarea of the lateral hypothalamic area.
LM: lateral mammillary nucleus
LPOA4: lateral preoptic area 4
MML: medial mammillary nucleus, lateral division
MMM: medial mammillary nucleus, medial division
MMP: medial mammillary nucleus, posterior division
mfb: medial forebrain bundle
mol: molecular layer of the lateral hypothalamus
mt: mamillo-thalamic bundle
mtt: mamillo-tegmental tract
OB: olfactory bulb
oc: optic chiasm
on: optic nerve
ot: optic tract
P: pons
PARH: para-arcuate hypothalamic nucleus

PFX: perifornical nucleus

POMA: magnocellular preoptic nucleus

PV1: parvalbumin-positive nucleus of the lateral hypothalamus

PV+d: parvalbumin-positive dendrite

PV+t: parvalbumin-positive terminal

RET: reticular nucleus of the thalamus

SIL: sublenticular substantia innominata

sm: medullary stria

TUA: area of the tuber cinereum

TUL: lateral tuberal nucleus (according to (Geeraedts et al., 1990a))

TUM1: part of the medial tuberal nucleus

TUMM: tuberomammillary nucleus

Type I: Gray type I synapse (asymmetric, excitatory)

Type II: Gray type II synapse (symmetric, inhibitory)

VMH: ventromedial hypothalamic nucleus

ZI: zona incerta

Neurotransmitters and neuromodulators:

MCH: melanocorticotropic hormone

5-HT: serotonin

Gal: galanin

NPY: neuropeptide Y

NT: neurotensin

TH: tyrosine hydroxylase

Most anatomical abbreviations are taken from the work of Geeraedts and colleagues (Geeraedts et al., 1990a) (Geeraedts et al., 1990b) on the cytoarchitecture of the medial forebrain bundle. Some other abbreviations are from the atlases of Swanson (Swanson, 2004) and Paxinos (Paxinos and Watson, 2009). The PV1-nucleus is a term proposed to describe the parvalbumin-positive neuronal aggregate described in this paper.

ABSTRACT

In the lateral hypothalamus, groups of functionally related cells tend to be widely scattered rather than confined to discrete, anatomically distinct units. However, using parvalbumin (PV)-specific antibodies, a solitary, compact cord of PV-immunoreactive cells (the PV1-nucleus) has been identified in the ventrolateral tuberal hypothalamus in various species. Here we describe the topography, the chemo-, cyto- and myeloarchitectonics as well as the ultrastructure of this PV1-nucleus in rodents. The PV1-nucleus is located within the ventrolateral division of the medial forebrain bundle. In the horizontal plane, it has a length of 1 mm in mice and 2 mm in rats. PV-immunoreactive perikarya fall into two distinct size categories and number ~800 in rats and ~400 in mice. They are intermingled with PV-negative neurons and coarse axons of the medial forebrain bundle, some of which are PV-positive. Symmetric and asymmetric synapses, as well as PV-positive and PV-negative fibre endings, terminate on the perikarya of both PV-positive and PV-negative neurons. PV-positive neurons of the PV1-nucleus express glutamate, not GABA - the neurotransmitter that is usually associated with PV-containing nerve cells. Although we could not find evidence that PV1 neurons express either catecholamines or known neuropeptides, they sometimes are interspersed with the fibers and terminals of such cells. From its analogous topographical situation, the PV1-nucleus could correspond to the lateral tuberal nucleus in humans. We anticipate that the presence of the marker protein PV in the PV1-nucleus of the rodent hypothalamus will facilitate future studies relating to the connectivity, transcriptomics, and function of this entity.

INTRODUCTION

The hypothalamus contains a dense and complex interweaving of cell groups and fiber pathways that has defied for more than a century the attempts of neuroscientist to identify its many components. Although the major cellular condensations in the medial hypothalamus were identified by (Gurdjian, 1927) and (Krieg, 1932), the lateral hypothalamic area (LHA) has proved much more difficult to subdivide. Saper and colleagues (Saper et al., 1979) divided the LHA rostrocaudally into four divisions: the lateral preoptic area, and the anterior, tuberal, and posterior LHA. These different rostrocaudal levels were found to have divergent patterns of projection as well.

The LHA is traversed by a longitudinal fiber pathway, the medial forebrain bundle, that connects the hypothalamus with the brainstem below it, and the basal forebrain and cerebral cortex above it. Veening and colleagues (Nieuwenhuys et al., 1982; Veening et al., 1982) divided the medial forebrain bundle into components based upon its myeloarchitecture and anterograde tracing from its known sites of origin. These studies underscore the complexity of the fiber pathways that run through the LHA and why electrical stimulation of the lateral hypothalamic area causes alterations in such disparate activities as feeding, sexual behavior, aggression, vocalization, as well as blood pressure and heart rate (Chi and Flynn, 1971; Hess and Akert, 1955; Hoebel and Teitelbaum, 1962; Lammers et al., 1988; Pfaff and Sakuma, 1979). The cellular bases for these responses are nearly impossible to interpret in such experiments.

The identification of chemically specified cell groups and their connections in the hypothalamus ushered in a new era in clarifying its structure and functions. For example, the identification of melanin concentrating hormone (MCH; (Bittencourt et al., 1992) and orexins (also known as hypocretins) (de Lecea et al., 1998; Sakurai et al., 1998), two peptide neurotransmitters that are expressed by neurons at the tuberal level of the LHA and in the dorsomedial and perifornical nuclei, has allowed identification of both discrete populations of neurons and their axonal pathways. In addition, the availability of genetically driven methods for

manipulating neuronal function (e.g., (Adamantidis et al., 2007) makes it tractable to study the function of chemically defined LHA cell populations. Interestingly, these two cell populations and their projection pathways are interlaced at virtually all levels, so that it would have been very difficult to distinguish these neuronal populations and their distinct physiological activities (Hassani et al., 2010; Lee et al., 2005; Mileykovskiy et al., 2005), and functions in wake-sleep and feeding (Chemelli et al., 1999; Kokkotou et al., 2005; Sapin et al., 2010) without this chemical distinction.

A great variety of neurotransmitters, neuropeptides, (Meister, 2007; Swanson, 1987; Swanson et al., 2005) and receptors (Kilduff and de Lecea, 2001) have been localized in neurons of the lateral hypothalamus, especially in rats and mice (Berthoud and Munzberg, 2011). But the chemical phenotypes of some neurons in the lateral hypothalamus remain unknown. An earlier publication (Celio, 1990) reported the presence of a continuous strand of parvalbumin-immunoreactive (PV-ir) neurons in the ventrolateral hypothalamus in rats. As virtually nothing was known about the connections or functions of this cell group, in the present study we have endeavored to better characterize this cell group in both rats and mice, and to determine the nature of its afferents, using classical histology, immunohistochemistry and electron microscopy.

MATERIALS AND METHODS

Thirty-four adult Wistar rats [275-300g (Janvier SAS, Le Genest-St-Isle, France)], 28 adult C57/Bl6 mice [20-30g (own breeding colony)] and 4 Balb-C mice [20-30g (Pathology Unit, Fribourg)] of both sexes were used for this study. Animals of the same species ($n=2$ for each postnatal day) were euthanized 5, 6, 7, 8, 9, 10 and 12 days after birth. In addition to the rodents, we examined sections from the brains of animals of other species which had been euthanized under deep sodium pentobarbital [Nembutal = sodium pentobarbital] anesthesia for a variety of unrelated reasons. These included sections through the brains of bats ($n=4$, kindly supplied by Irmgard Amrein PhD, University of Zürich, Switzerland,

experiments approved by the Veterinarian commission of the Canton of Zürich); cats ($n=2$, euthanized by Benoit Deillon, DVM, Bulle, for medical causes), guinea pigs ($n=2$, kindly supplied by Alexandre Babalian PhD, Physiology Dept. University of Fribourg, euthanized for neurophysiologic experiments approved by the Veterinary Commission of the Canton of Fribourg); and cynomolgous monkeys ($n=5$, kindly supplied by Grazyna Wieczorek DVM, Novartis AG, Basel, euthanized for kidney transplantation experiments, approved by the Veterinary commission of the Canton of Basel Stadt). The overall study was approved by the Veterinary Commission for Animal Research of the Canton of Fribourg, Switzerland. All animals were housed in state of the art animal facilities according to the strict Swiss animal testing law. In preparation for perfusion, the rodents were anaesthetized with a mixture of Ketalar [(Parke-Davis) 75 mg/kg of body weight] and Xylazine [(Streuli) 10 mg/kg of body weight]. A 0.9% saline solution followed by a 4% paraformaldehyde solution in 0.1M phosphate buffer (pH 7.4) were perfused through the left ventricle. The brains were excised and maintained in 18% or 30% solution of sucrose in phosphate buffered containing 0.1% sodium azide for one day at 4°C. They were then frozen in pulverized dry ice. The specimens were cryosectioned into 40- or 80- μ m-thick sections and collected in 0.1 M phosphate buffer (pH 7.3). Immunofluorescence- and immunoperoxidase-staining techniques were conducted according to published protocols (Celio, 1990; Gerig and Celio, 2007). Briefly, free floating sections were first incubated with one of the primary antibodies to parvalbumin, which were used at dilutions of 1:5'000 - 1: 10'000. All of the antibodies used are listed in Table 2, and their characterization is described in the next section. After treatment with a biotinylated secondary antibody, the sections were exposed either to streptavidin CY2, Cy3 or Cy5 (Jackson immunohistochemicals) for immunofluorescence or to the avidin-biotin-peroxidase complex (Vector laboratories, Burlingame, Ca, USA) for immunohistochemistry. This last complex was revealed using diaminobenzidine-hydrogen peroxide as the substrate. Immunostaining for PV was combined with that for well-characterized calcium binding-proteins, neuropeptides, neurotransmitters and catecholamines antisera.

To stain the perineuronal net, Texas Red-conjugated *Wisteria-floribunda* lectin (WFA) was used (prepared by incubating the dye with the lectin at a concentration of 20 µg/ml in TRIS-buffered saline (0.1 M, pH 7.3) containing 0.1% Triton-X, 0.1mM MgCl₂, 0.1mM MnCl₂ and 0.1mM CaCl₂).

GAD-antibodies (gift of Dr. W. Oertel and acquired from Chemicon/Millipore) as well as monoclonal antibodies against GABA and glutamate (Swant Inc., Marly, Switzerland) crosslinked to albumin using glutaraldehyde (diluted 1:500) were dissolved in a solution of Na-pyrophosphate and Na-borohydride in TRIS-buffered saline, together with mono- or polyclonal antibodies against PV [(Swant) 1:500 dilution] and tested on free-floating, 40- µm-thick coronal sections (Celio, 1986b). The secondary antibodies were Alexa®-488-conjugated anti-rabbit IgG and Alexa®-567-conjugated anti-mouse IgG [(Invitrogen) diluted 1:500] for 4 hours at ambient temperature.

Antibody Characterization

Antibodies against parvalbumin (235, PV28 and PVG214, all Swant Inc., Marly, Switzerland) recognize only one band of 12 Kda MW in Western blots of brain extract in various species, rat and mice included. They stain the brain in a pattern consistent with previous results (Celio, 1990) and do not produce staining in the brain of parvalbumin knock-out mice (Schwaller et al., 1999).

Antibodies against calbindin D-28k (300, CB38, both Swant Inc., Marly, Switzerland) recognize only one band of 28 kDa MW on immunoblots of brain extract in various species, rat and mice included. At very high antibody concentration CB38 may cross-react with calretinin. Both antibodies stain in a pattern consistent with previous studies (Celio, 1990) and are negative on the brain of calbindinD-28k knock-out mice (see product specification sheet of Swant Inc.).

Calretinin antibodies (6B3, CR6797, both Swant Inc., Marly, Switzerland) recognize only one band of appropriate MW (29 KDa) in an immunoblot of brain extract in various species, rat and mouse included. They are distributed in a pattern of cellular morphology and distribution as in previous studies (Rogers and

Résibois, 1992) and do not stain the brain of calretinin knock-out mice (see product specification sheet of Swant Inc.).

GAD-antibodies were kindly provided by Dr. Wolfgang Oertel (Oertel et al., 1981) or acquired commercially (Chemicon, now Millipore see Table). These last react with a protein of the expected molecular weight in immunoblots of rat and mouse brain extracts and stain in a pattern consistent with results published by others (Mugnaini and Oertel, 1985; Wu et al., 1986).

GABA (3D5, 3A12) and Glutamate monoclonal antibodies, antibodies (2D7), both of Swant Inc., Marly, Switzerland, were produced respectively against a glutaraldehyde-linked GABA- or glutamate-BSA conjugate (Liu et al., 1989; Matute and Streit, 1986). In dot blots they react with the appropriate amino acid, do not cross react with other amino acids (Celio, 1986a) and in glutaraldehyde-fixed tissue, their staining patterns are consistent with results by others using similar antibodies (Somogyi et al., 1985; Storm-Mathisen et al., 1983) (Seguela et al., 1984).

Hypocretin / Orexin A (H-003-30) and B (H-003-32) antibodies were acquired from Phoenix Pharmaceuticals (Burlingame, Ca). They were produced against synthetic peptides with no crossreaction with similar peptides and their staining can be inhibited by absorption with the adequate antigen (1 $\mu\text{g}/\mu\text{l}$). Their staining pattern is consistent with previous localization results (de Lecea et al., 1998).

Light microscopy and quantification

The sections were examined in either a Zeiss photomicroscope equipped for fluorescence with a SPOT-camera (Mod. 11.1), a Nikon 6000 photomicroscope equipped with a digital camera (DXM 1200) or a Leica confocal laser-scanning microscope (TCS Sp5 / DM 6'000). Brightness, contrast and evenness of illumination of the images were adjusted with Adobe Photoshop (CS4).

The numbers of neurons in the PV1-nucleus were counted on free-floating, 40- μm -thick horizontal serial sections through the brains of rats (Wistar: $n=6$) and mice (C57Bl/6: $n=6$; Balb-C: $n=4$) of both sexes. All PV-immunoreactive neurons in which the nucleus (4 μm) was visible in a DAPI-stain (Invitrogen) were counted with the aid of ImageJ software and the counts corrected by the method for estimation of the "true" cell number in the investigated brain area (Guillery and Herrup, 1997) .

Electron microscopy

Rats were perfused with a solution of 4% paraformaldehyde. Coronal vibratome sections through the brain (50 μm in thickness) were exposed to PV-28 antibodies (Swant, Marly, Switzerland) for 72 hours at 4°C, followed by the avidin-biotin-peroxidase technique. The specimens were postfixed in Karnovsky's solution (a mixture of 2.5% glutaraldehyde and 2.5% paraformaldehyde) and then stained with 1% OsO₄. After being embedded in Epon, the region containing the PV1-nucleus was excised and affixed to an empty block of resin for sectioning. Inspection of the first 0.5- μm -thick semi-thin section in the light microscope served to establish the orientation and topography of the PV1-nucleus. Ultrathin sections were then prepared using a Reichert ultramicrotome, stained with uranyl citrate and viewed in an EM-100 transmission electron microscope (Philips Biotwin CM100).

RESULTS

Overview

The PV1-nucleus is a bilateral, elongated condensation of cells, which extends longitudinally and almost horizontally through the lateral hypothalamus. This slender cord of cells has a length of approximately 2 mm in rats [spanning bregma levels -2.04 to -4.16 (Paxinos and Watson, 2009)] and approximately 1 mm in mice [spanning bregma levels -1.15 to -2.18 (Franklin and Paxinos, 1997)]. Three to ten PV-ir cells are visible in the cross-section of the PV1-nucleus. The best view of the PV1-nucleus is seen in horizontal sections, which reveal it to be sandwiched between the fornix medially, and the outer pial surface and the optic tract laterally (Fig. 1). Rostrally it fans out and coalesces with PV-positive neurons in the magnocellular preoptic area. Caudally, the PV1-nucleus borders the lateral mammillary nuclei and the PV-immunoreactive tuberomammillary nucleus, from which it is clearly demarcated.

Under conditions of dark-field illumination, the cross-sectioned PV-1 nucleus is revealed as a myelin-rich region (Fig. 2A). In sections that have been stained with Cresylviolet, only a small number of nerve cells can be discerned in the PV1-nucleus, which is barely recognizable as a discrete entity (Fig. 2B). The dimensions of the PV1 cell group and the number of cells per section is similar to the A1 noradrenergic group in the medulla (see, e.g., (Tucker et al., 1987)).

The number of PV-ir cells in the PV1-nucleus (counted bilaterally) amounts to 850 +/- 45 cells in adult Wistar rats ($n=6$) and to 360 +/- 40 and 370 +/- 54 in adult Balb-C ($n=4$) and C57/BL6 mice ($n=6$), respectively. From time to time some animals show fewer cells in the PV1-nucleus, an as yet unexplained phenomenon. No right-left differences or sexual dimorphism is apparent on casual inspection, but our study was not powered to provide a meaningful statistical evaluation of these features.

PV-immunoreactive cells are first discerned in the lateral hypothalamus during the second postnatal week, around day 6 +/- 1 in rats and day 5 +/- 1 in C57 BL/6

mice. At the beginning the cells are roundish and in the adult become elongated or multipolar.

In bats, guinea pigs and cats, a small collection of a few PV-ir neurons was observed in the region corresponding to the position of the PV1-nucleus in rats and mice (not shown). In rabbits, monkeys and humans, parvalbumin-immunoreactive neurons were not detected in the ventrolateral region of the hypothalamus, which corresponds in position to the location of the PV1-nucleus in rats and mice.

Precise localization of the PV1-nucleus

In coronal sections, the position of the PV1-nucleus changes slightly in the rostrocaudal direction (Fig. 3A and 3B). It begins rostrally at the level of the anterior hypothalamic area, where it is located dorsolateral to the supraoptic nucleus, its coordinates in rats at this level are [-2.04mm from bregma (-1.15 in mice)], -8.8 mm from the dura (-4.8 in mice), and 2.2 mm from the midline (+/- 1.4 in mice) (Fig. 3, B&B'). See also Table 1. Caudally, the PV1-nucleus in rats shifts slightly more medially, its coordinates at this level being -4.16 mm anteroposteriorly (-2.18 in mice); -8.8 ventral to the dura (-4.8 in mice) and +/- 2.0 mm from the midline (+/- 1.1 in mice) (Fig. 3, D&D'; see also Table 1). The ventromedial bend in the PV1-nucleus occurs where the supraoptic decussation and the optic tract pass beneath the internal capsule, which extends to the lower surface of the brain (viz., to bregma levels -3.24 to -3.60 in rats).

In coronal sections, four topographically distinct portions of the nucleus can be distinguished. The first lies just caudal to the medial preoptic area, lateral and cranial to the most lateral tip of the optic tract (3B, 3B'). The second portion is adjacent to the optic tract, which passes beneath the PV1-nucleus in a medial-to-lateral direction (Fig. 3C & 3C' and 3D & 3D'). The third portion lies in the angle formed between the optic tract and the internal capsule (Fig. 3E & 3E'). The fourth, more caudal and deepest portion sometimes bulges at the base of the brain (Fig. 3, F & F').

Relationship of the PV1-nucleus to hypothalamic blood vessels

The rostral, middle and caudal portions of the PV1-nucleus are either traversed by, or closely associated with blood vessels that penetrate the hypothalamus from the base of the brain (Figs. 1D, 3B' and 6H).

General morphology of the PV1-nucleus and its neurons

In horizontal sections (Fig. 1) the PV1-nucleus appears as a strand of fairly compactly organized small and medium-sized PV-immunoreactive neurons, which lie amidst axons of the medial forebrain bundle, some of which are also PV-positive (Figs. 1C & 1D). The numerical density of cells is higher in the rostral than in the caudal part of the PV1-nucleus. Small neurons are encountered more frequently in the rostral and mid regions, and larger ones in the caudal part (Fig. 1D). In sequential parasagittal and horizontal sections, and depending somewhat on the inclination of the slice, the PV1-nucleus has a fragmented appearance, although it is always distinguishable as a compact assembly of PV-immunoreactive cells. In the PV1-nucleus, PV-immunoreactive neurons are often oriented horizontally and have a bi- or multipolar form. Their nuclei are large (6 μm diameter) and chromatin poor and show a distinct nucleolus (Fig. 4, A, B and D). The perikarya are either small (15-20 μm in diameter) or middle-sized (25-30 μm in diameter). The smaller cells are usually bipolar (along the anteroposterior axis), whereas the larger ones are either fusiform or multipolar (Fig. 1, inset). The dendrites of the PV-immunoreactive neurons extend to the pial surface laterally and to the perifornical region medially. In the PV1-nucleus of mice, middle-sized neurons predominate over smaller ones. In both species, immunoreactivity for PV varies greatly in intensity; but, as a general rule, smaller cells tend to be less strongly stained than larger ones. PV-immunoreactive nerve fibers form boutons near PV-negative (Fig. 4 C, D) perikarya, irrespective of their size, but PV-ir boutons also form appositions along both the cell bodies and dendrites of PV-positive neurons, usually the larger ones (Fig. 4 A & 7 E,F). Some PV-ir terminals on large cells may derive from small cells and *vice versa*, thus indicating intranuclear connections inside the PV1. The region of the PV1-nucleus that is

rich in PV-immunoreactive perikarya is characterized by a higher numerical density of PV-positive terminals (Fig. 4 A, C) and of variously sized axons emanating from the medial forebrain bundle (Fig. 1D). Fine PV-ir axons, which may stem from neurons in the PV1-nucleus, are sometimes seen to attain the pial surface or to course medially. However, most of the PV-immunoreactive axons leaving the PV1-nucleus proceed either rostrally or caudally via the medial forebrain bundle. Some of the axons leaving the PV1-nucleus proceed in a dorsolateral direction, following the course of the supraoptic decussation.

Other markers of the PV1-nucleus

We scanned a library of brains from previous studies for other neurotransmitters in the region of the PV-1 cell aggregate, but were unable to find evidence for the presence in that cell group of neurons immunoreactive for thyrotropin releasing hormone, orexin (Chou et al., 2003); galanin (Gaus et al., 2002); tyrosine hydroxylase, choline acetyltransferase, melanin-concentrating hormone, adenosine deaminase, histamine (Chou et al., 2002); dynorphin (Chou et al., 2002); neurotensin, corticotropin releasing hormone, brain natriuretic peptide, somatostatin, enkephalin, (Moga and Saper, 1994); adrenocorticotrophic hormone, α -melanocyte stimulating hormone (Moga et al., 1990); or endomorphin (Greco et al., 2008) (see also Fig. 5.).

In particular, because PV is often seen in GABAergic neurons, we were especially surprised not to find any evidence for neurons that were immunoreactive for glutamic acid decarboxylase (Sherin et al., 1998) in the PV1 cell region. The only potential neurotransmitter that we found in this area immunohistochemically was glutamate (Chou et al., 2003).

We also looked for evidence of mRNA for these same neurotransmitters in the PV1 cell group, in both our own preparations for glutamic acid decarboxylase 67 and galanin (Chou et al., 2003; Gaus et al., 2002), as well as in the Allen Brain Atlas database for the other markers we examined immunohistochemically, and of these only the vesicular glutamate transporter 2 (Chou et al., 2003) was present in region harboring the PV1 cell group.

We therefore did double-labeling for glutamate and for PV, and confirmed that nearly all of the PV-1 neurons are glutamatergic (see Fig. 6 A-C). Of the tested neuropeptide antisera, those against MCH and hypocretin-orexin A and B stained neurons in close vicinity to the PV1-nucleus, and some axon terminals ended in the cell cluster of the PV1 (Fig. 6D).

Two other calcium binding proteins (calbindin D-28k and calretinin) are richly represented in the hypothalamus but generally avoid the PV1-nucleus. Nevertheless, some scattered bipolar cells that were immunoreactive for either CB or CR were found within the boundaries of the PV1 cell group (Fig. 6 E-F).

Markers that are known to be associated with either the membranous cytoskeleton or the outer surface of PV-immunoreactive neurons in other parts of the brain were demonstrated also in the PV1-nucleus. These markers included those of the membranous cytoskeleton (Ankyrin-R and Spectrin-R) (not shown) and of the extracellular perineuronal net (the *Vicia-villosa* and *Wisteria-floribunda* lectins) (Fig. 6G).

Ultrastructure of neurons in the PV1-nucleus

At the ultrastructural level, the small neuronal perikarya of the PV1-nucleus exhibit an ovoid form. They possess an indented nucleus and are rich in rough endoplasmic reticulum. Perikarya and dendrites of PV-positive cells are involved in the formation of both symmetrical and asymmetrical synapses (Fig. 7A - 7 D). They are also contacted by PV-positive endings (Fig. 7 E, F). Some of these parvalbumin-positive terminals may emanate from neurons in the PV1, thus being intranuclear contacts.

DISCUSSION

The findings of the present study identify the PV-1 cell group as a clear cytoarchitectonic and neurochemical entity in rats and mice, although it could not

be detected in all species we have studied. The PV-ir neurons are apparently glutamatergic, which is quite unusual for PV neurons in mammals.

Comparison with previous studies

The cell density of the PV1 nucleus, particularly the rostral part, is higher than that in the neighboring lateral hypothalamic area. Therefore, this cell group can be named as a „nucleus“, not like a part or just a cell group, or a subdivision of the lateral hypothalamic area, as described by those having recognized these cells previously (Bleier et al., 1979; Geeraedts et al., 1990b).

In a series of landmark publications, Nieuwenhuys and his co-workers (Geeraedts et al., 1990a; Geeraedts et al., 1990b; Nieuwenhuys et al., 1982) reported on the existence within the lateral hypothalamus of a thick bunch of myelinated nerve fibers stemming from the medial forebrain bundle. Axons comprising the dorsolateral portion of this so-called “a-bundle” were postulated to derive from the olfactory tubercle, the magnocellular preoptic nucleus and the nucleus of the lateral olfactory tract, whereas those comprising its ventromedial portion were believed to stem from the anterior lateral hypothalamic area, the ventral part of the lateral septal nucleus and the nucleus of the diagonal band (Geeraedts et al., 1990a; Geeraedts et al., 1990b).

Cytoarchitectonically, the region embracing the “a-bundle” was referred to by the same group of investigators as the ventrolateral subarea of the lateral hypothalamus (LHVL1) (Geeraedts et al., 1990a; Geeraedts et al., 1990b). Coronal and sagittal sections through the LHVL1 revealed the presence of a small and circumscribed cluster of cells – referred to as “nucleus-2” in single coronal and sagittal sections – which almost certainly corresponds to the middle portion of the PV1-nucleus described here (Fig. 3, A&A'). Swanson (Swanson, 2004) name a somewhat larger rectangular volume the parvicellular region of the lateral hypothalamic area (LHAapc).

PV-positive neurons are not scattered over the entire cross-sectional surface of the so-called “a” bundle, but are concentrated within its lower half. They represent only one – albeit a preponderant – subpopulation of nerve cells in the

PV1-nucleus. Rostrally, the PV-immunoreactive cord transgresses the boundaries of the LHV1 (Geeraedts et al., 1990a; Geeraedts et al., 1990b; Nieuwenhuys et al., 1982) and coalesces with cells in the sublenticular substantia innominata and interstitial nucleus of the stria terminalis (Geeraedts et al., 1990a; Geeraedts et al., 1990b). In other atlases, this region coincides approximately with the magnocellular preoptic area (Paxinos and Watson, 2009), also called lateral hypothalamic area, anterior region, ventral zone (Swanson, 2004). Caudally, the PV1-nucleus is confined to the LHV1 and is clearly demarcated medioventrally from the terete nucleus (Paxinos and Watson, 1999) and the mammillary nuclei.

A cell group corresponding to the PV1-nucleus (Celio, 1990) now appears in atlases of the mouse (Hof et al., 2000) and rat brain (Paxinos and Watson, 2009), albeit under another name – the preteretis nucleus – in the latter case. However, the PV1 runs parallel to the terete nucleus, and is clearly separated and cytoarchitectonically distinct from it.

PV-immunoreactive nerve cells in cortical areas are often GABA-expressing interneurons (Celio, 1986b). However, the region of the lateral hypothalamus which embraces the PV1-nucleus has been observed to contain neither the GABA-synthesizing enzyme GAD (Mugnaini and Oertel, 1985) nor its mRNA (www.brain-map.org), even though GABA-positive axons terminate in the region. Given that PV-positive cells of the PV1-nucleus (i) manifest immunoreactivity for glutamate, (ii) probably express the mRNA for VGlut2 [according to the Allen Database (www.brain-map.org)], and (iii) are subject to retrograde filling with tracers injected into putative target areas in the midbrain (Celio and Saper, 1999), it seems likely that most, if not all, of the PV-immunoreactive cells in the PV1-nucleus are glutamate-expressing projecting neurons. It remains to be ascertained if the large PV-positive neurons in the caudal part of the PV1-nucleus project to the cortical mantle, as it has been shown for neuron of similar size and shape, located along the medial edge of the cerebral peduncle (Saper, 1985).

Cytology, cytoarchitectonics and cell counting

Our observations relating to the cytology and cytoarchitectonics of neurons in the PV1-nucleus accord well with the findings reported by Nieuwenhuys and his colleagues (Geeraedts et al., 1990a; Geeraedts et al., 1990b; Nieuwenhuys et al., 1982; Veening et al., 1982). We likewise observed a bipolar orientation of the smaller neurons and an increase in cell size rostrocaudally. In addition, we have reported (i) on the existence of two neurochemically distinct cell types (small and large PV-positive neurons as well as PV-negative ones), (ii) quantified the number of PV-immunoreactive neurons, (iii) and did not find evidence for right-left asymmetry or sexual dimorphism in the numbers of PV1 neurons. The number of PV-immunoreactive neurons in the PV1-nucleus of rodents is about half of that estimated for hypocretin/orexin - expressing cells in C57/BL6 mice (Brownell and Conti, 2010; Gardi et al., 2008; Pinos et al., 2011).

An age-dependent decline in the number of PV-immunoreactive neurons can be observed in the PV1-nucleus of mice between the 24th and 32nd postnatal months (www.brain-map.org). But further studies will be needed to ascertain whether this finding is attributable to a loss of immunoreactivity for PV or to the death of PV-expressing neurons.

Relationship of the PV1-nucleus to hypothalamic blood vessels

The rostral end of the PV1-nucleus coincides with the level at which the small lateral hypothalamic vessels enter the hypothalamus (e.g. Fig. 6H). The vessels that are seen either to traverse or to pass close to the PV1-nucleus are probably the medial and posterior fascicular arteries and veins (Ambach and Palkovits, 1979). But whether the relationship is of topographic interest alone or of physiological relevance remains to be clarified.

Neurotransmitters, neuromodulators and calcium binding proteins

We tested for more than 20 different putative neurotransmitters and neuropeptides in the PV1 neurons, but found only glutamate to be present. While it is possible that the use of colchicine injections might have identified

some other neurotransmitter in the PV1 cell group, the absence of anything but glutamate immunoreactivity in that population in intact rats and mice is quite striking. MCH- and orexin-immunoreactive neurons were located very close to the PV1 (e.g. Fig 6G, H) but respected the boundaries of the PV1 field. In some cases immunoreactive axons, most notably for tyrosine hydroxylase, serotonin and cholecystinin, coursed through the PV1-nucleus, and immunoreactive terminals formed appositions with PV1 neurons. Rare neurons positive for calbindin D-28k and calretinin mixed in with the PV1 group, but no colocalization with PV was observed (Figs. 6E-F). Orexin and MCH-positive neurons came close but neither occupied the field of the PV1, nor coexisted with PV in the same cell (Figs. 6D).

Is the rat PV1-nucleus homologous to the human lateral tuberal nucleus?

The lateral tuberal nucleus was described microscopically by Le Gros Clarke (Le Gros Clark, 1938) as a distinctive group of small neurons embedded in a gelatinous neuropil along the ventrolateral border of the hypothalamus in human brains. These neurons were found to stain immunohistochemically for a variety of neuropeptides, including somatostatin, and corticotropin-releasing factor (Timmers et al., 1996). A homologous-appearing structure has been identified in some non-human primates (Narkiewicz et al., 1994). A lateral tuberal nucleus has not been identified in rodents (Gurdjian, 1927); (Krieg, 1932); (Franklin and Paxinos, 1997; Paxinos and Watson, 1999; Swanson, 2004). In more recent publications on the rat brain (Geeraedts et al., 1990a; Geeraedts et al., 1990b), the lateral tuberal nucleus has been depicted as a region lying dorsomedially to the LHVl, in which we localize the PV1-nucleus (TUL in their Fig 36; (Geeraedts et al., 1990b). In 1979 however, Bleier and her colleagues described on the basis of cytoarchitectonic criteria a "lateral tuberal nucleus" in the rat hypothalamus (Bleier et al., 1979) whose position corresponds to that of the PV1-nucleus. Indeed, at least the caudal part of the PV1-nucleus in rodents occupies a similar position to the lateral tuberal nucleus in monkeys (Fujii, 1982) and humans (Le Gros Clark, 1938). These observations raise the possibility that the lateral tuberal

nucleus in primates might be represented by the PV1-nucleus in rodents. However, the lateral tuberal nucleus in monkeys and humans lacks immunoreactivity for PV (Celio, unpublished observations), which cannot therefore be used as an argument in favor of a postulated homology. Nevertheless, phylogenic differences in the expression of calcium-binding proteins are not uncommon, the best known examples being the expression of calbindin D-28k and secretagoin in Meynert's basal nucleus only in primates (Celio and Norman, 1985; Mulder et al., 2010).

Hence, alternative criteria must be implemented to confirm or refute the postulated homology – perhaps an expression pattern for a common set of genes.

Derivation and development

In studies relating to the expression of Foxb1 in the developing ventral diencephalon, Alvarez-Bolado et al. (Alvarez-Bolado et al., 2000) identified patches of hypothalamic neuroepithelium that gave rise to neurons which migrate over long distances to colonize the ventrolateral nucleus of the lateral hypothalamic area (Geeraedts et al., 1990b). The authors remarked that the final destination of the labeled cells did not precisely coincide with any of the previously described hypothalamic nuclei, and they therefore referred to this niche for neurons as “Foxb1-expressing cells of the lateral hypothalamic nucleus”. The location of this cluster of Foxb1-expressing cells corresponds closely to that of the rodent PV1-nucleus (compare their figures 3A and 4A with our Fig. 1C). Although Foxb1 is expressed mainly by large neurons on the 21st postnatal day, it is absent from the nerve cells of adult animals (www.brain-map.org). It would therefore be interesting in the future to determine if the Foxb1 neurons also express PV in young animals. Alternatively, a Foxb1-Cre mouse could be crossed with a green fluorescent protein (GFP) reporter mouse, producing permanent staining of the transiently Foxb1-positive neurons. This would allow localization of the GFP with respect to parvalbumin, and could aid in tracing the projections of these neurons.

PV-ir cells are first discerned in the PV1-nucleus of rodents around the 6th postnatal day, concomitant to their colonization of the cerebral cortex. In the Allen Brain Atlas, the expression of PV is discerned between the 7th and the 21st postnatal days. Immunoreactivity for hypocretin, on the other hand, appears in the lateral hypothalamus on about the 15th postnatal day (Yamamoto et al., 2000).

Possible functions of the PV1-nucleus

Although the LHA has been implicated in older electrical stimulation or lesion studies in a wide range of behaviors, from regulation of sleep (Arroyo et al., 1993; Economo, 1930; Jurkowlanec et al., 1996), to feeding (Delgado and Anand, 1953; Turenius et al., 2009), and blood pressure control (Hess, 1981; Spencer et al., 1989), it is difficult if not impossible to determine whether the PV1 neurons played a role in these responses. Similarly, while the sites used for classical studies on intracranial self-stimulation were also nearby (Olds and Fobes, 1981), it is difficult to know whether PV1-neurons were activated during intracranial self-stimulation, let alone playing a role in sustaining it.

In the history of hypothalamic research, clinical observations on patients have often prepared the ground for understanding the role of various parts of this intricate brain region. Adiposogenital dystrophy (Fröhlich, 1901), due to a tumor in the region of the arcuate nucleus foreshadowed the role of this nucleus in the control of appetite. Narcolepsy is explained today as a disorder of loss of signaling by hypocretin/orexin neurons (Saper et al., 2010). Within or close to the lateral wall of the tuberal region of the human hypothalamus hamartomas arise and lead to gelastic (*laughing*) seizures (Arroyo et al., 1993; Kuzniecky et al., 1997; Valdueza et al., 1994). The lateral hypothalamus has also been implicated in the proper functioning of the larynx (Arita et al., 1995) and the trachea (Tatsuta and Arita, 1996) and in the control of vocalization (Burgdorf et al., 2007). The neurons that compose hypothalamic hamartomas have been found to be mainly GABAergic and many contain gonadotropin-releasing

hormone (Chan et al., 2010), so they are not likely to arise from the PV1 cell group. However, tumors in this region may potentially irritate a human homologue of the PV1-nucleus. It is therefore tempting to suppose that the PV1-nucleus may be involved in primitive forms of vocalization, found also in rodents (Panksepp and Burgdorf, 2003). Perhaps genetically-driven electrophysiological or optogenetic methods that are defined by the presence of parvalbumin in the nucleus may provide a way to test this hypothesis.

Nowadays, it is rare to discover aggregates of nerve cells with a common biochemical signature in the brain. Assuming that each brain nucleus has a specific role to play, the search for a possible functional implication of the hypothalamic PV1-nucleus becomes a thrilling challenge.

FIGURE LEGENDS

Fig. 1

Topography of the PV1-nucleus in the lateral hypothalamus

A: Ventral view of a perfusion-fixed rat brain. The approximate location of the PV1-nucleus is highlighted with two red stripes and indicated with an arrow. See list of abbreviations. Scale bar: 1 mm.

B: Schematic tracing of a horizontal section at the level of the PV1 nucleus, reproduced in modified form from (Geeraedts et al., 1990b). For abbreviations see table 1. The projected full extent of the PV1 nucleus is highlighted in blue. The contour of the PV1 at this level (compare with Fig. 1C) is given by the stippled red line.

C: Horizontal section through the rat hypothalamus at a depth of 8.4 mm (Paxinos and Watson, 2009). The PV1-nucleus is oriented parallel to the fornix (fx), located medial to the optic tract (ot) and separated from the brain surface by the molecular layer (mol). The PV1-nucleus – outlined with a broken white line –

is here seen to contain a large number of PV-immunoreactive neurons. The anterior part is rich in small-, the posterior in large neurons (see also Fig. 1D). See list of abbreviations. Scale bar: 1 mm.

D: Higher-magnification view of the PV1 from another experiment (immunoperoxidase staining). In this horizontal plane of section the distinction between the rostrally located, smaller PV-positive neurons (S) and the larger neurons located distally (L) is well visible. The gap between the two cell populations is simply due to the particular inclination of the plane of section. A blood vessel (V) is found in proximity to the small cell cluster. Scale bar: 0.5 mm.

Fig. 2

The PV1 in dark-field and cresyl violet stained sections

A: Dark-field image of the lateral hypothalamus. The PV1-nucleus is lodged within a bundle of myelinated nerve fibers, which lies dorsal to the supraoptic commissure and the optic tract (ot).

B: Bright-field images of the lateral hypothalamus (stained with cresyl violet). The PV1-nucleus is revealed as a cell-poor region, most of the neurons being located between the myelinated fibers. Scale bar: 0.25 mm.

Fig. 3 A and 3B

The PV1- nucleus on coronal sections

A-F (left): schematic representations and A'-F' (right): light micrographs of the PV1-nucleus, as seen in a series of 6 coronal sections with a spacing of approximately 200 μm . The PV-immunoreactive cell-cluster is located in the LHV1 that is described by (Geeraedts et al., 1990b), and precisely coincides with their "subnucleus-2" at level T11 in their cross section (corresponds to level D of this figure). In B'-F', the PV1-nucleus is encircled, and is represented at higher magnifications in the insets. Note differences in the numerical density of the PV-immunoreactive cells at the different levels. In its most anterior part the PV1-nucleus is intimately associated with the optic tract and lies along the medial

edge of this nerve-fibre bundle (B', E'). In its most caudal part, the PV1-nucleus is exposed and sometimes bulges at the lower surface of the brain (F'). In the insets B', and E' profiles of vessels are seen along the periphery or crossing the PV1-nucleus. The schemes have been reproduced with modifications from (Geeraedts et al., 1990a; Geeraedts et al., 1990b). See list of abbreviations
Scale bar: 1 mm.

Fig. 4

Confocal laser-scanning microscopy of PV-positive neurons

PV-immunoreactive neurons (A) and DAPI-stained cell nuclei (B) in the PV1-nucleus. The nuclei of the PV-immunoreactive nerve cells are chromatin-poor and harbor a distinct nucleolus. PV-positive endings terminate on the perikarya and on the dendrites (lines ending in a circle) of PV-immunoreactive cells. Parvalbumin-positive endings encroach on the surface of three adjacent, parvalbumin negative cells bodies (C, arrows) with typical neuronal nuclei (D, arrows). Scale bar: 20 μ m

Fig. 5

PV1 lacks neurotransmitters and neuromodulators positive cell bodies

Immunolabelling for four different neuropeptides and two catecholamines in the lateral hypothalamus. The absence of MCH- (A), neurotensin (NT: B), galanin (Gal: C) expressing neurons from the PV1-nucleus is striking. The numerical densities of NPY-(D) and 5HT-positive (F) fibers and endings are extremely low. The PV1-nucleus is particularly rich in TH-positive fibers. Note the presence of a small blood vessel along the periphery of the PV1-nucleus (in A, B and C). See also list of abbreviations. Scale bar: 0.3 mm.

Fig. 6

Parvalbumin cells of the PV1-nucleus are positive for glutamate and negative for other calcium binding proteins and hypocretin / orexin

Neurons of the PV1-nucleus manifest immunoreactivity for both glutamate (A) and PV (B), as is evident in the merged image (C: yellow coloration represents dual labeling). Hypocretin/orexin perikarya are scattered in close proximity to the PV1, send terminals between parvalbumin positive neurons, but never coexist with parvalbumin in the same neuron (D). Cells positive for calbindin D-28k (E) and calretinin (F) are sometimes found inside the perimeter of the PV1 as defined with PV-antibodies, but no coexistence can be observed. R: rostral, M: medial, L: lateral. Scale bar for A, B and C 50 μm . Scale bar for D, E and F: 100 μm .

The perineuronal net of extracellular matrix is observed around the cell body of large parvalbumin neurons of the PV1 (G). Scale bar: 50 μm .

Blood vessel arborisation in close proximity to the rostral part of the PV1 (H). Scale bar: 0.5 mm

Fig. 7

Transmission electron micrographs of axon terminals in the PV1-nucleus.

Both probable Gray type II (A and B; inhibitory) and type I (C, D and E; excitatory) synapses impinge on the surface of parvalbumin-positive dendrites (PV-d). Some synapses cannot be classified. Terminals impinging on PV-immunoreactive profiles in the PV1-nucleus can be either PV-negative (A-E) or PV-positive (PV+t); F). Scale bars in A, C and D: 0.1 μm ; in B: 0.05 μm ; in E and F: 0.2 μm .

Fig. 8

Three-dimensional reconstruction of the PV1-nucleus from serial coronal sections.

In the rat, the PV1-nucleus (red string) is represented as a long, slender accumulation of PV-immunoreactive cells in the lateral hypothalamus. Near its centre, the nucleus bends slightly ventrally. The PV1-nucleus is intimately associated with the optic tract (ot), the internal capsule (yellow) and the lower

surface of the brain. In the mouse, the PV1-nucleus has a more cucumber-like form (not shown). See also list of abbreviations. Scale bar: 1 mm.

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Table 1: Location of the PV1-nucleus in rats and mice

Species	Rat (Wistar, 300 g)		Mouse (C57/Bl6, 25 g)	
	Anterior	Posterior	Anterior	Posterior
Anteroposterior	-2.04	-4.16	-1.15	-2.18
Lateral	+/- 2.2	+/- 2.0	+/- 1.4	+/- 1.1
Depth	8.8	8.8	4.8	4.8

Legend to Table 1: Coordinates of the PV1-nucleus in rats and mice. The values are derived from the atlas of Paxinos and Watson (Paxinos and Watson, 1999; Paxinos and Watson, 2009)

Table 2: **Antibodies used in this study**

Antibodies and antisera	Immunogen	Producer or catalogue and lot no.	Clonality and dilution
GAD 67 (Gamma-amino-decarboxylase)	Recombinant GAD 67 protein	Chemicon Cat. no. AB5992 (clone 1g10.2) and Dr. W. Oertel (Oertel et al., 1981)	Mouse monoclonal and goat polyclonal 1:1'000 to 1:3'000
Parvalbumin	Purified carp PV (235); purified rat PV (PV28 and PVG 214)	Swant 235, PV28, PVG214	Mono- and rabbit + goat polyclonals 1:5'000 to 1: 20'000
Calbindin D-28k	Calbindin D-28k purified from chicken gut (300) and recombinant rat calbindin D-28k (CB 38)	Swant 300, CB38	Mono and rabbit polyclonal 1:5'000 to 1: 20'000
Calretinin	Recombinant human calretinin	Swant 6B3, CR7699	Mono- and rabbit polyclonal 1:5'000 to 1: 20'000
GABA	GABA-glutaraldehyde-bovine serum albumin	Swant 3D5 and 3A12	Monoclonals 1:500
Glutamate	Glutamate-glutaraldehyde-bovine serum albumin	Swant 2D7	Monoclonal 1:500
Orexin A and B	Synthetic peptide	Phoenix Pharmaceuticals H-003-30 and H-003-32	Polyclonal (rabbit) 1:1'000

Legend to Table 2: Antibodies utilized in this study. Some commercial antibodies have been characterized in scientific publications (e.g., those sold by Swant Inc.) information respecting which accompanies the company's description of the product.

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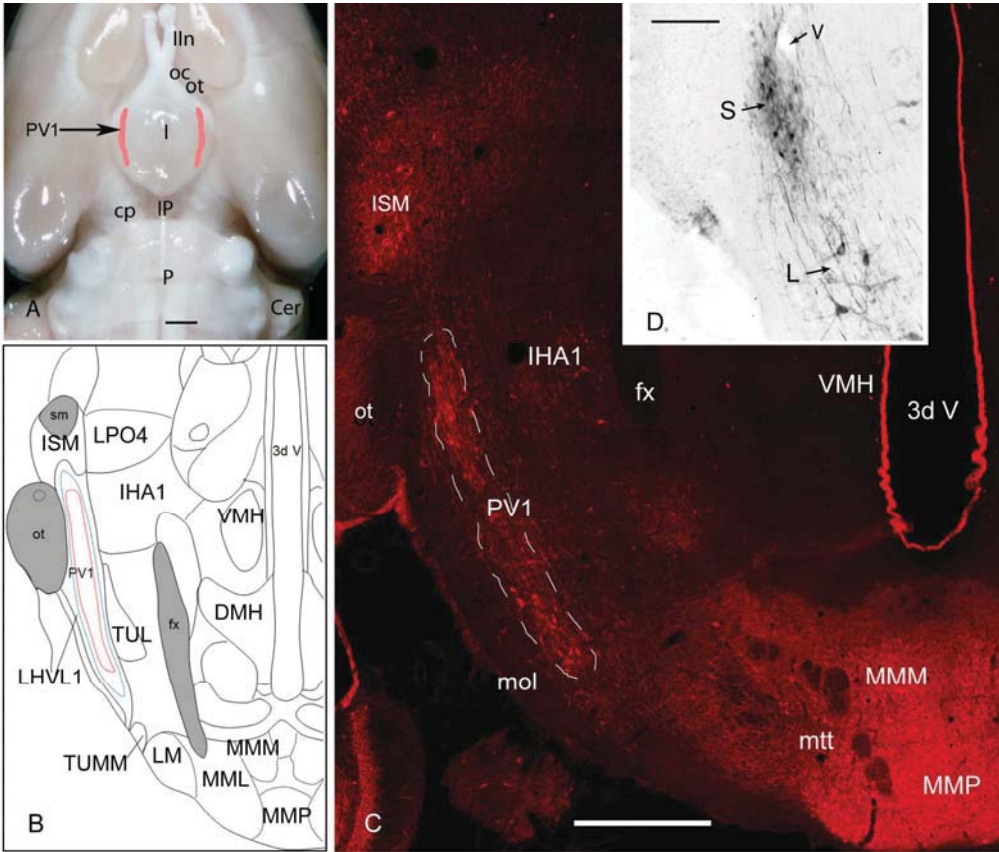
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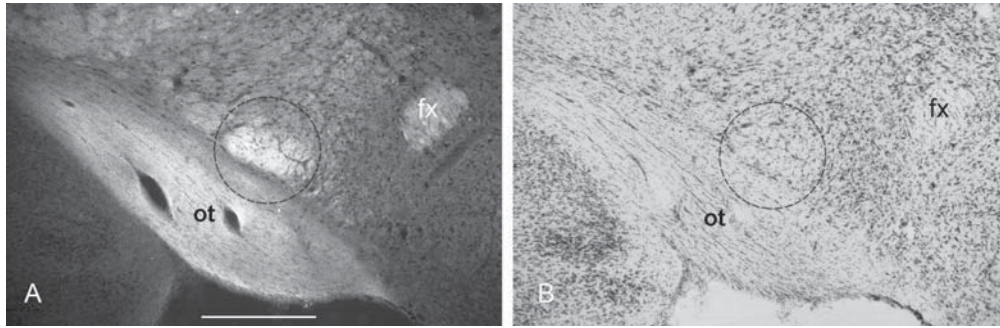
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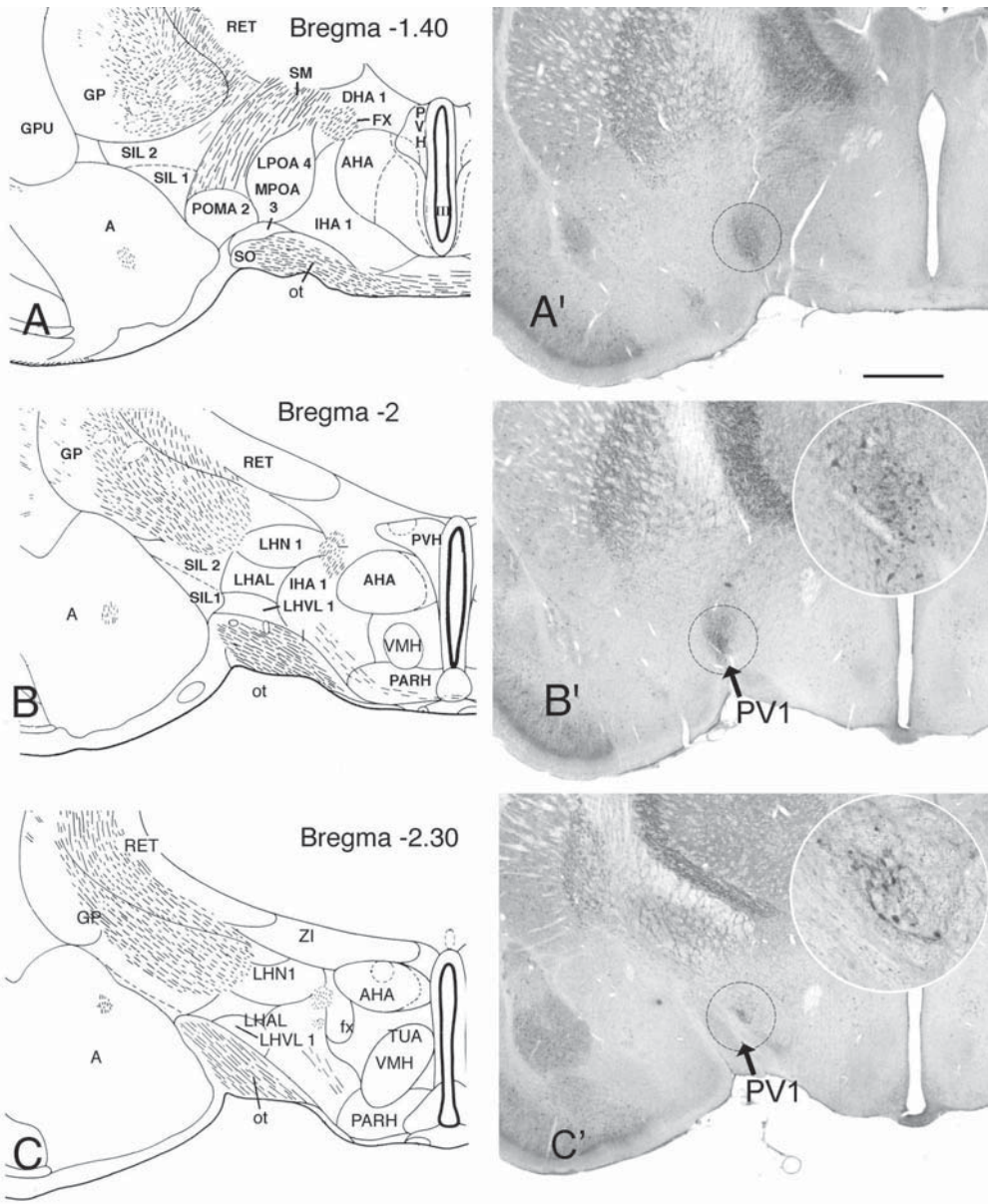
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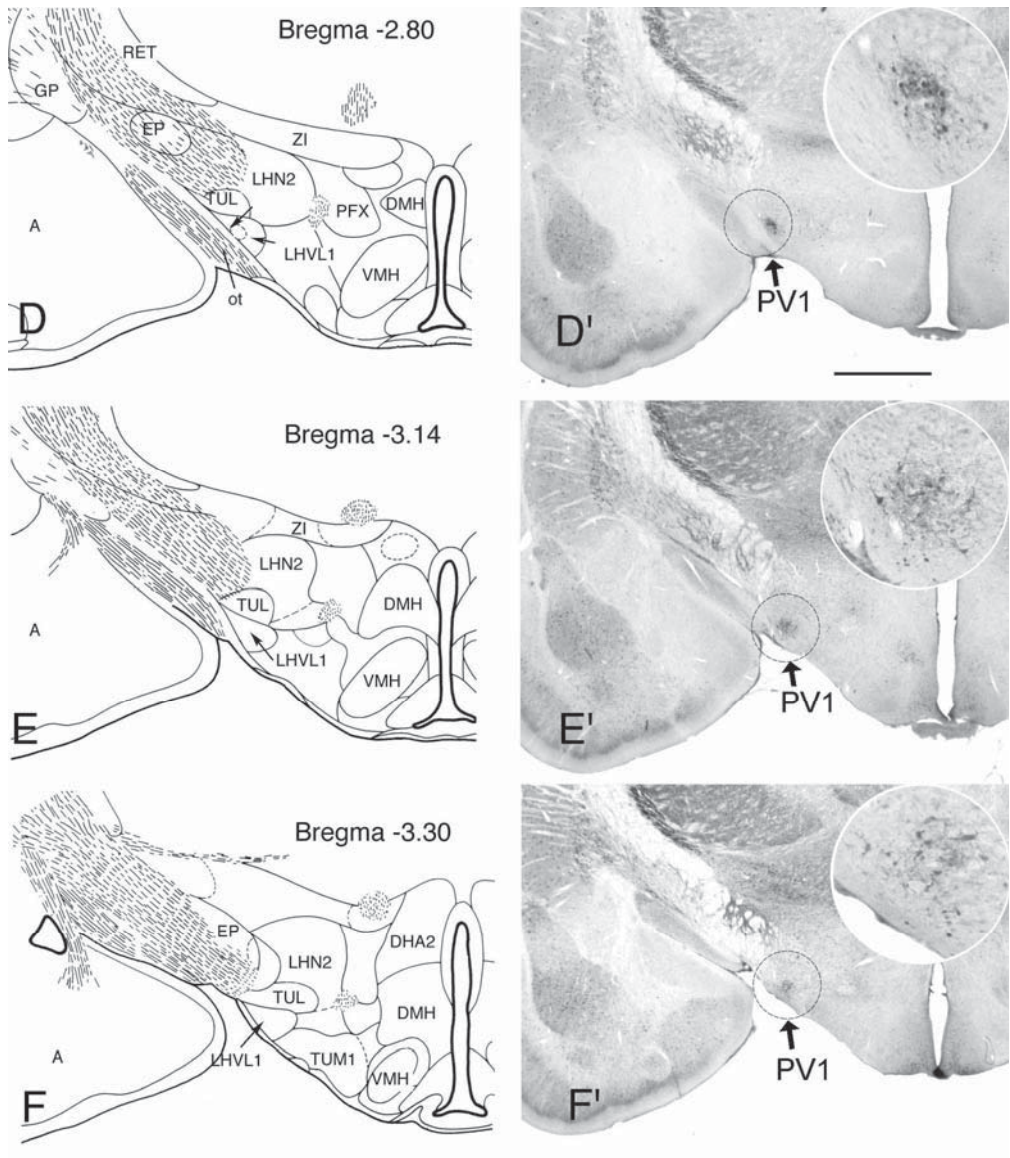
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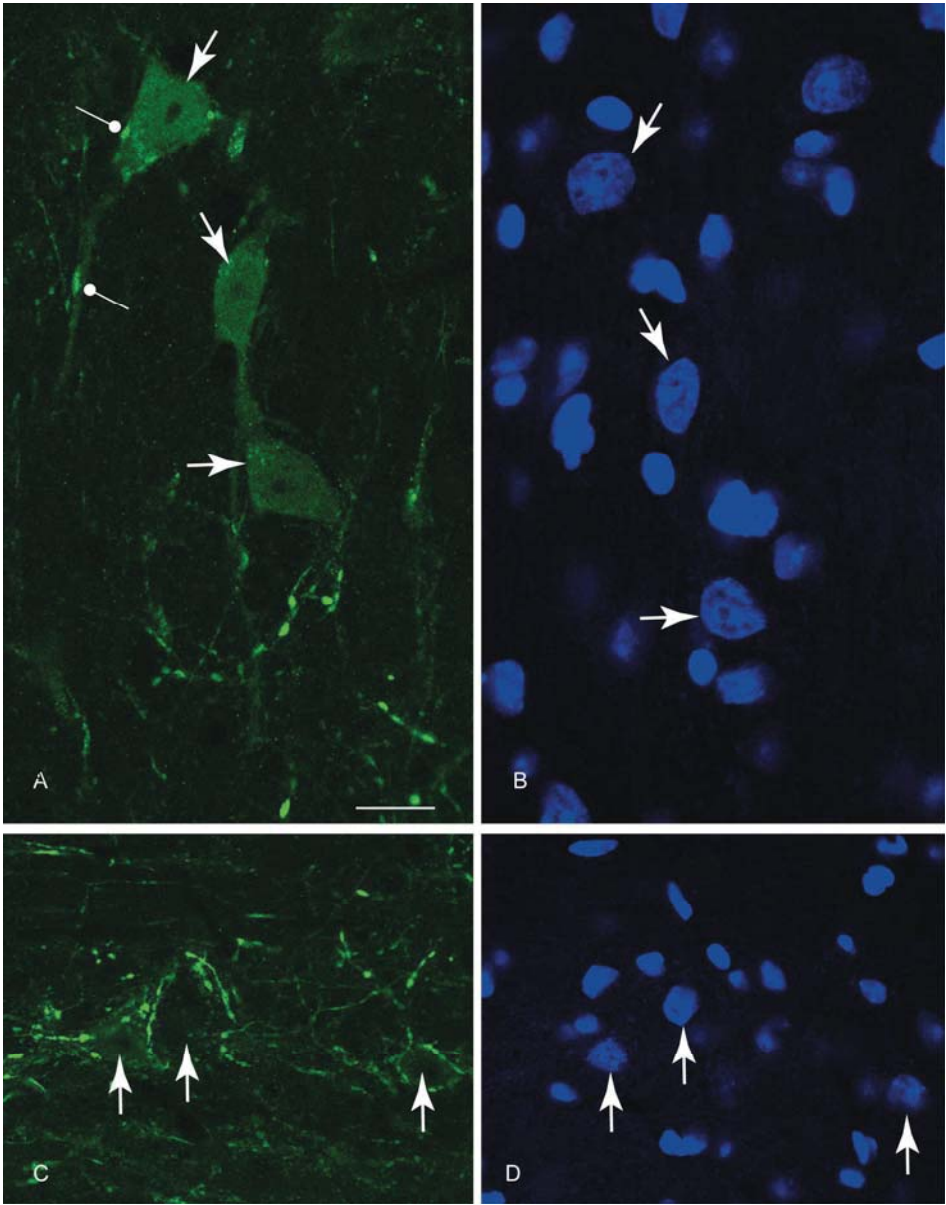
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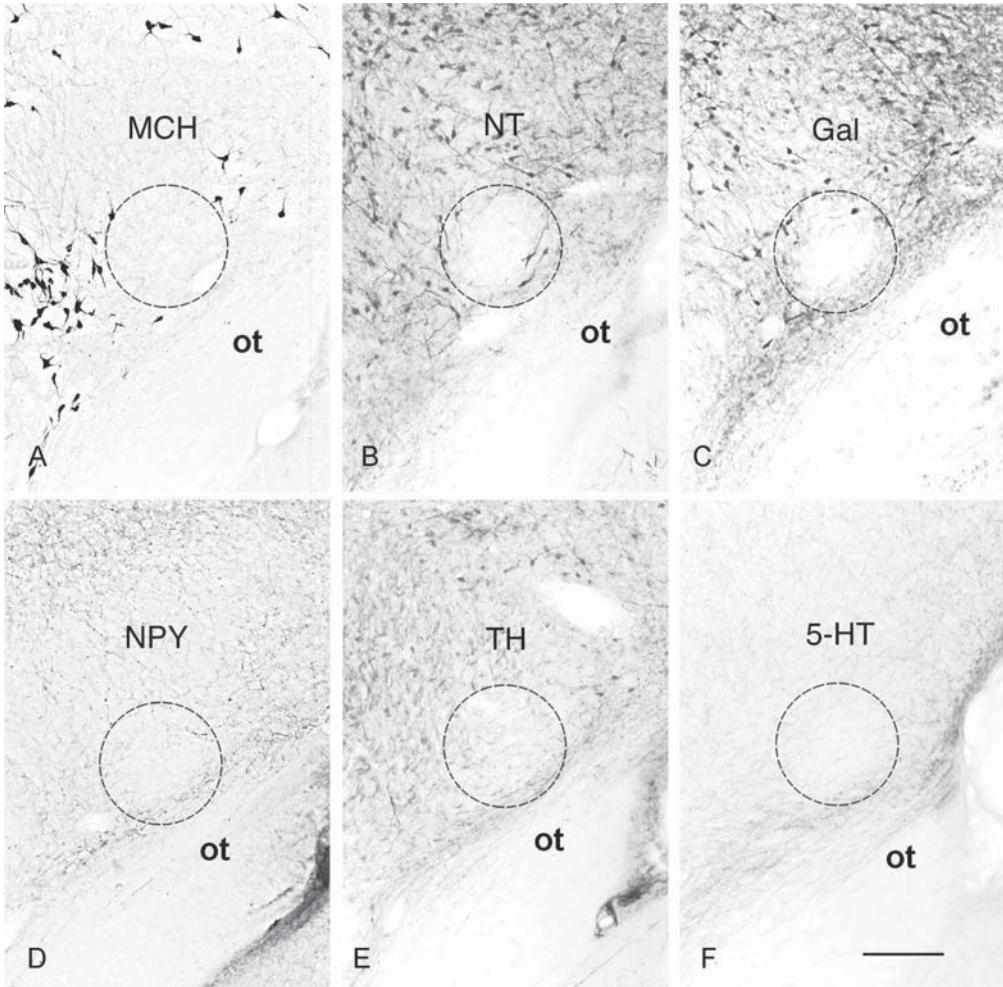
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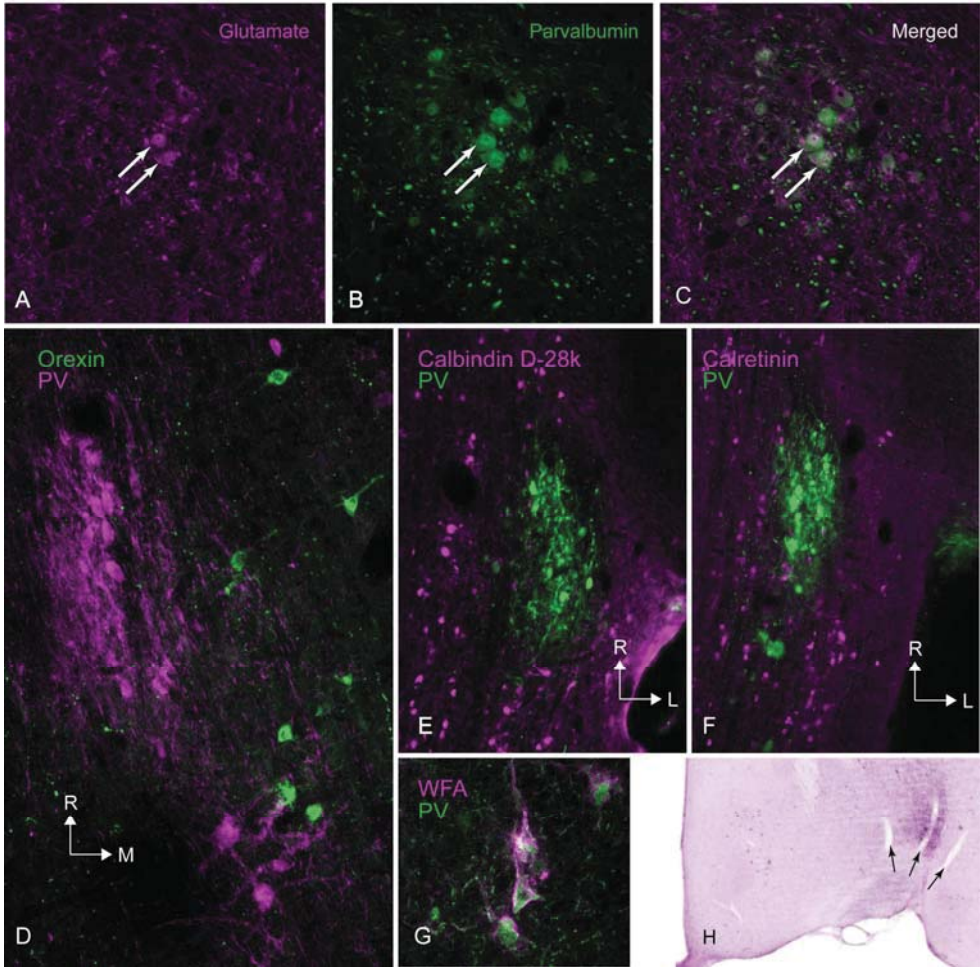
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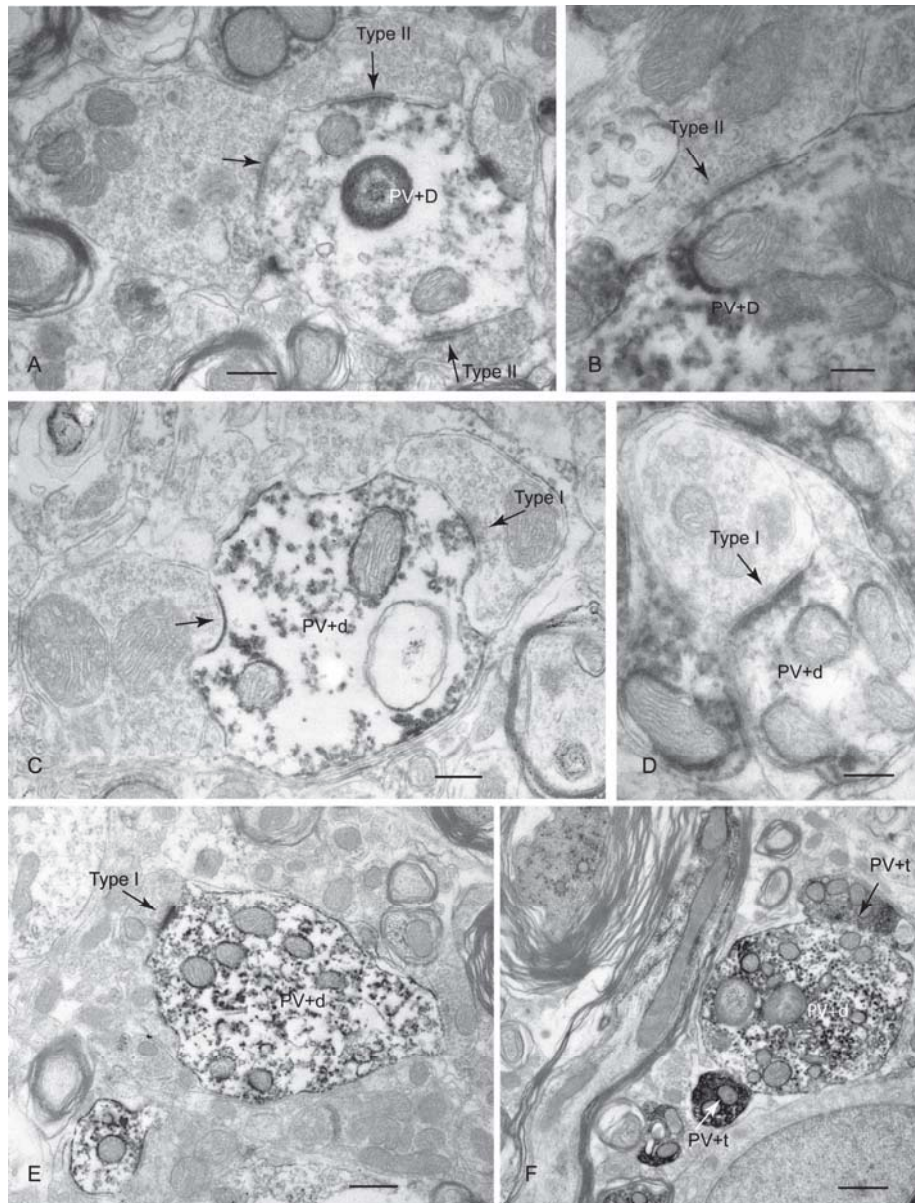
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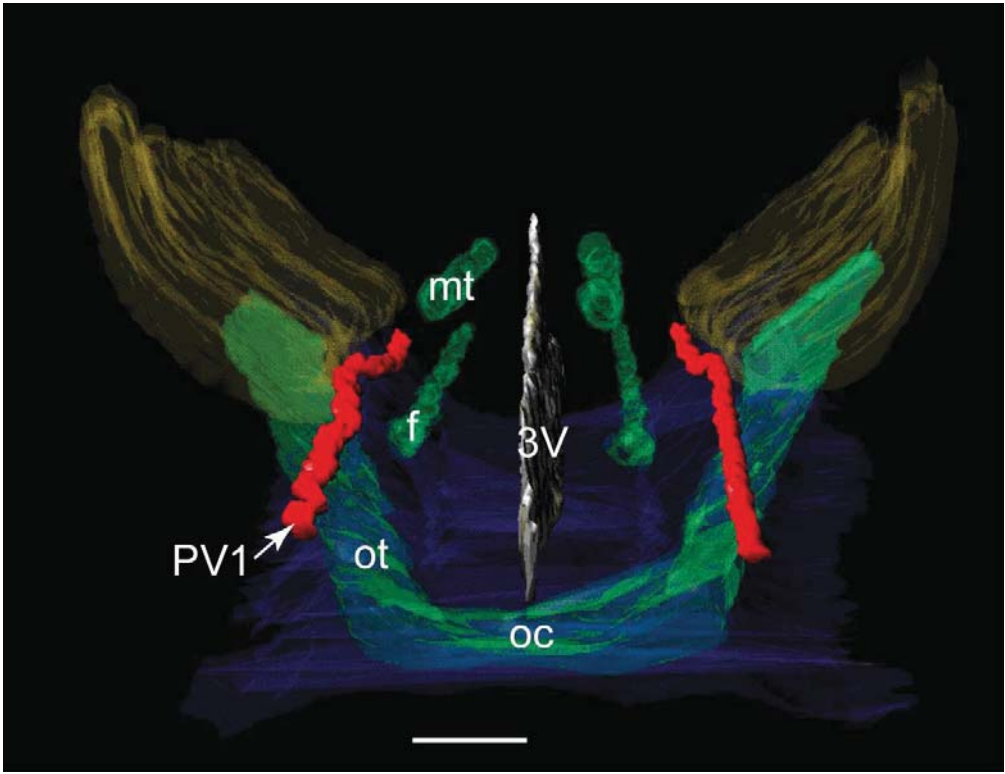
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